

Appl. No. : 10/045,185  
Filed : October 18, 2001

### REMARKS

Claims 1-8 and 11-20 have been cancelled. Claim 9 has been amended. Claims 9 and 10 are now pending in this application. Support for the amendments is found in the existing claims and the specification as discussed below. Accordingly, the amendments do not constitute the addition of new matter. Applicant respectfully requests the entry of the amendments and reconsideration of the application in view of the amendments and the following remarks.

#### **Rejection under 35 U.S.C. § 112, first paragraph**

Claims 1 and 9 are rejected under 35 U.S.C. § 112, first paragraph, because the specification, while being enabling for enhancing the survival of memory T cells after exposure to an antigen with IL-15, does not reasonably provide enablement for treating an immune deficiency disease (such as HIV) by administering anti-IL-15 antibodies.

Applicants believe the rejection of claim 1 to be a typographical error as claim 1 was cancelled with the amendment filed on October 18, 2001.

To address the rejection of claim 9, Applicants have amended claim 9 to recite a method of treating an autoimmune disease by administering "an anti-IL-15 antibody or a fragment of said antibody to a mammal in need thereof, when the auto-immune response is subsiding in order to suppress formation of memory cells of the immune system". Support is found in the substitute specification at page 5, last paragraph to page 6, first full paragraph (corresponding to paragraphs 0016 & 0017 of the published application) and in cancelled claims 11 and 18 and previous claim 9.

The specification clearly teaches that the immune system can be inhibited from returning to an activated state by administration of antibodies to IL-15. That is, an unwanted or harmful CD4+ T-cell dependent immune response such as may be present in an autoimmune disease may be inhibited by antibodies to IL-15. Suppression and/or regression of autoreactive CD4+ effector T-cells results in beneficial effects for the patient.

Further support for this approach is found in Matthys, et al. (Attachment A) which shows that treatment with anti-IL-15 antibodies increases apoptosis of thymocytes. CD4+CD8+ subpopulations were most severely effected (see Table 1). This effect could be reversed by administration of IL-15 (Figure 3). This further supports regression of CD4+ memory cells by administration of antibodies to IL-15.

**Appl. No.** : **10/045,185**  
**Filed** : **October 18, 2001**

The Examiner asserts that Grabstein, et al. indicate that in an immune deficiency condition such as HIV infection, it is desirable to activate the immune response by administering IL-15. This point is moot in view of Applicants' claim amendment to recite that the method is directed to treatment of an autoimmune disease, not HIV. One skilled in the art would be able to administer anti-IL-15 antibodies as claimed to a patient suffering from an autoimmune disease. Evidence that the claimed invention could be successfully obtained is found in the specification and the Matthys, et al. reference as discussed above.

In view of Applicants' amendments and arguments, reconsideration and withdrawal of the above ground of rejection is respectfully requested.

**Rejection under 35 U.S.C. § 102(b)**

Claims 9-11, 14, and 18 are rejected under 35 U.S.C. § 102(e) as being anticipated by Grabstein, et al. (U.S. Patent No. 5,795,966).

The Examiner asserts that the intended use is given no weight and that Grabstein, et al. disclose antibodies against IL-15.

While Grabstein, et al disclose antibodies against IL-15, Grabstein, et al. do not disclose the use of antibodies against IL-15 for the same purpose. Grabstein, et al. teach inhibition of T-cell stimulation and proliferation (see Grabstein, et al. col. 1, lines 6 to 10; column 12, lines 23-25). In contrast, Applicants' invention teaches establishment of a resting phenotype in T-cells without driving the T-cells into proliferation (see substitute specification at page 17, paragraph 1 through page 18, paragraph 1 which correspond to published application at paragraphs 0064 to 0066).

While Grabstein, et al. do not teach a specific therapy for treatment of an autoimmune disease, the therapy of Grabstein, et al is potentially useful during the acute phase of an autoimmune disease. The acute phase occurs before an actual immune response takes place and can be treated by blocking T-cell proliferation. By contrast, antibodies to IL-15 according to the claimed invention are administered "when the auto-immune response is subsiding in order to suppress formation of memory cells of the immune system" (claim 9 as amended). This limitation is not taught by Grabstein, et al. That is, the presently claimed invention is directed to administration of antibody to IL-15 during the chronic phase of an auto-immune disease, not the

Appl. No. : 10/045,185  
Filed : October 18, 2001

acute phase. Administration of the IL-15 antibody promotes regression/suppression of memory cells thereby reducing the possibility of relapses.

In view of Applicants' amendments and arguments, reconsideration and withdrawal of the above ground of rejection is respectfully requested.

**CONCLUSION**

In view of Applicants' amendments to the claims and the foregoing Remarks, it is respectfully submitted that the present application is in condition for allowance. Should the Examiner have any remaining concerns which might prevent the prompt allowance of the application, the Examiner is respectfully invited to contact the undersigned at the telephone number appearing below.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated:

July 21, 2005

By:

Che S. Chereskin

Che Swyden Chereskin, Ph.D.

Registration No. 41,466

Agent of Record

Customer No. 20,995

(949) 760-0404

1821322  
071905

## ATTACHMENT A

FAST TRACK

## Induction of IL-15 by TCR/CD3 aggregation depends on IFN- $\gamma$ and protects against apoptosis of immature thymocytes *in vivo*

P. Matthys<sup>\*1</sup>, H. DOOMS<sup>†1,2</sup>, P. ROTTIER<sup>†</sup>, T. MITERA<sup>\*</sup>, L. OVERBERGH<sup>†</sup>, G. LECLERCQ<sup>§</sup>, A. BILLIAU<sup>\*</sup> & J. GROOTEN<sup>†</sup> *\*Laboratory of Immunobiology, Rega Institute for Medical Research and †Laboratory for Experimental Medicine and Endocrinology (LEGENDO), University of Leuven, Leuven, ‡Department of Molecular Biomedical Research, Molecular Immunology Unit, Flanders Interuniversity Institute for Biotechnology and Ghent University, Ghent and §Department of Clinical Chemistry, Microbiology and Immunology, University Hospital, Ghent University, Belgium*

(Accepted for publication 11 September 2002)

### SUMMARY

TCR/CD3 aggregation by injection of anti-CD3 Ab produces T cell activation, release of cytokines such as IFN- $\gamma$  and apoptosis in the cortical region of the thymus. We show that anti-CD3 Ab induces IL-15 mRNA in spleens of wild-type but not IFN- $\gamma$  receptor-knock-out (IFN- $\gamma$ R KO) mice. The loss of IL-15 mRNA induction in IFN- $\gamma$ R KO mice was associated with increased thymocyte apoptosis. Pretreatment of wild-type mice with neutralizing anti-IL-15 Ab increased the anti-CD3-triggered thymocyte apoptosis, thus mimicking the sensitive phenotype of IFN- $\gamma$ R KO mice. Inversely, anti-CD3-induced apoptosis in IFN- $\gamma$ R KO mice was suppressed by administration of recombinant IL-15. In IFN- $\gamma$ R KO mice and in wild-type mice that were treated with anti-IL-15, augmented apoptosis affected mainly CD4<sup>+</sup>CD8<sup>+</sup> immature thymocytes. IL-15 as well as IL-15R $\alpha$  mRNA expression in thymocytes was not increased by anti-CD3. These data demonstrate that systemic IL-15 exerts anti-apoptotic activity on immature T cells and establish a regulatory mechanism whereby TCR/CD3 engagement induces IL-15 expression via an IFN- $\gamma$ -dependent pathway. The self-amplifying nature of this IFN- $\gamma$ /IL-15 connection may constitute a regulatory pathway in central tolerance to self.

**Keywords** Knockout mice T lymphocytes apoptosis autoimmunity

### INTRODUCTION

Antibodies against the TCR/CD3 membrane complex of T lymphocytes are being used as an experimental tool to polyclonally stimulate T lymphocytes in a way resembling 'natural' oligoclonal stimulation by antigens presented to the TCR by APCs. The response of T cells to anti-CD3 triggering depends on their stage of maturation. Mature T cells, stimulated in the presence of APC-derived costimulatory signals, respond by proliferating. In the absence of costimulatory signals they either become anergic or undergo apoptosis, a response which is considered to constitute

the main mechanism of peripheral tolerance. In immature T lymphocytes, as a contrast, exposure to anti-CD3 Ab invariably results in apoptosis, and this response is considered to reflect the physiological elimination of self-reactive T lymphocytes in the thymus, also known as negative selection, accounting for central tolerance.

Injection of anti-CD3 Ab is a system in which the mechanism and regulation of TCR/CD3-triggered T lymphocyte apoptosis can be analysed. Both in man and in mice, the injection results in a massive release of various cytokines, including IFN- $\gamma$  and TNF- $\alpha$ , and in an acute shock-like reaction designated as the anti-CD3 syndrome [1–4]. However, another feature of the reaction is extensive apoptosis in the cortical region of the thymus [5]. Previously, we observed that IFN- $\gamma$ R KO mice are more sensitive to the anti-CD3 syndrome and exhibit more extensive apoptosis in the thymus than their wild-type counterparts [4]. This indicated that IFN- $\gamma$ , produced in the course of the syndrome, exerts an anti-apoptotic effect, either directly or, more likely, by inducing or facilitating production of other cytokines.

Correspondence: Dr P. Matthys, Rega Institute, University of Leuven, Minderbroedersstraat 10, B-3000 LEUVEN, Belgium.  
E-mail: Patrick.Matthys@rega.kuleuven.ac.be

<sup>1</sup>P.M. & H.D. contributed equally to this work. <sup>2</sup>Current address: Department of Pathology, University of California at San Francisco, 513 Parnassus Avenue, San Francisco, CA 94143–0506.

One anti-apoptotic cytokine is IL-15. The mRNA of this cytokine is constitutively expressed in various tissues; the protein is produced by mononuclear phagocytes, epithelial cells and some others, but not by T or B lymphocytes [6,7]. IL-15 uses the  $\beta$  and  $\gamma$  chains of the IL-2 receptor and its biological activities resemble those of IL-2: it stimulates proliferation of NK, T and B lymphocytes, and augments cytotoxicity of T cells and Ab production by B cells [6,8–11]. We have recently reported that IL-15, unlike IL-2, exerts strong anti-apoptotic activity on mature T cells [12,13]. Thus, IL-15 protected mouse CD4<sup>+</sup> T cells from TCR-induced apoptosis, resulting in either a proliferative response or anergy, depending on the presence or absence of costimulatory signals. Also, T cells from mice expressing an IL-15 transgene were found to be protected against activation-induced cell death [14]. *In vivo* administration of an IL-15-IgG2b fusion protein protected splenocytes as well as hepatocytes from anti-Fas-induced apoptosis [15]. In addition, IL-15 was found to protect fibroblasts against TNF- $\alpha$ -induced cell death [16].

Clearly, IL-15 modulates apoptosis induced by various triggers in a wide variety of cells. However, although apoptosis is a prominent feature of immature T cells undergoing negative selection in the thymus, no reports on a possible role of IL-15 in the apoptotic response of thymocytes to TCR/CD3 triggering are available. To examine this question, we used *in vivo* induction of thymocyte apoptosis by injection of anti-CD3 Ab in mice as a model. We measured expression of IL-15 mRNA in wild-type and IFN- $\gamma$ R KO mice, taking advantage of their being relatively resistant or sensitive, respectively, to the apoptotic response, and we studied the effect of a neutralizing anti-IL-15 Ab on the extent of thymocyte apoptosis.

## MATERIALS AND METHODS

### Mice and reagents

IFN- $\gamma$ R KO (129/SV/Ev) [17] and wild-type mice were bred under specific pathogen-free conditions in our Experimental Animal Breeding Facility. Experiments were done with 7–8-week-old mice. The monoclonal antibody, directed against the mouse CD3 complex (clone 145–2C11), was prepared as described [4]. Monoclonal neutralizing antibody against mouse IL-15 (clone G277-3588) was obtained from Pharmingen (San Diego, CA, USA). Irrelevant rat IgG1 isotype control antibody (control IgG) was obtained as described [18]. Batches of all antibodies were found to contain less than 1 ng/ml endotoxin (KabiVitrum, Stockholm, Sweden). Human recombinant IL-15, that is active on murine cells, was obtained from R & D Systems (Abingdon, UK).

### In vivo experiments

Age- and sex-matched IFN- $\gamma$ R KO and wild-type mice were injected i.p. with 100  $\mu$ l saline containing 0, 10, 20 or 40  $\mu$ g of anti-CD3 Ab. In some experiments, mice were pretreated with anti-IL-15 Ab or control rat IgG (0.4 mg in 100  $\mu$ l saline, i.p.) five hours before anti-CD3 challenge. IL-15 was injected i.p. (15  $\mu$ g in 100  $\mu$ l PBS) at 6 h and 18 h post anti-CD3 challenge. Control animals received two injections of 100  $\mu$ l PBS. At the indicated time points, mice were sacrificed and spleens or thymuses were obtained for either RNA extraction or flow cytometric analysis.

### Real-time PCR

RNA was extracted using TRIzol Reagent (Life Technologies, Gaithersburg, MD, USA). cDNA was synthesized using a

TaqMan® Reverse Transcription Reagent kit (Roche Molecular Systems, Inc., Branchburg, NJ, USA). Real-time quantitative PCR was performed in the ABI prism™ 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA). The PCR reactions for mL-15 and  $\beta$ -actin were performed as described [19] using a dually labelled fluorogenic probe. Briefly, PCR amplifications were done in a total volume of 25  $\mu$ l, containing 0.5  $\mu$ l cDNA sample, 1  $\times$  Taqman buffer A, 200  $\mu$ M dNTPs, 5 mM MgCl<sub>2</sub>, 100–300 nM of each primer, 100 nM probe and 0.625 U AmpliTaqGold (Applied Biosystems). All PCR amplifications were performed in triplicate under the following conditions: 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The  $\beta$ -actin mRNA was used as an internal standard to control variability in amplification. The level of mL-15 mRNA, relative to  $\beta$ -actin mRNA, was calculated using the formula:

$$\text{Relative mRNA expression} = 2^{-(\text{Ct of target} - (\text{Ct of } \beta\text{-actin} + 25))}$$

Ct is on the threshold cycle value shown as the mean of three different PCR reactions.

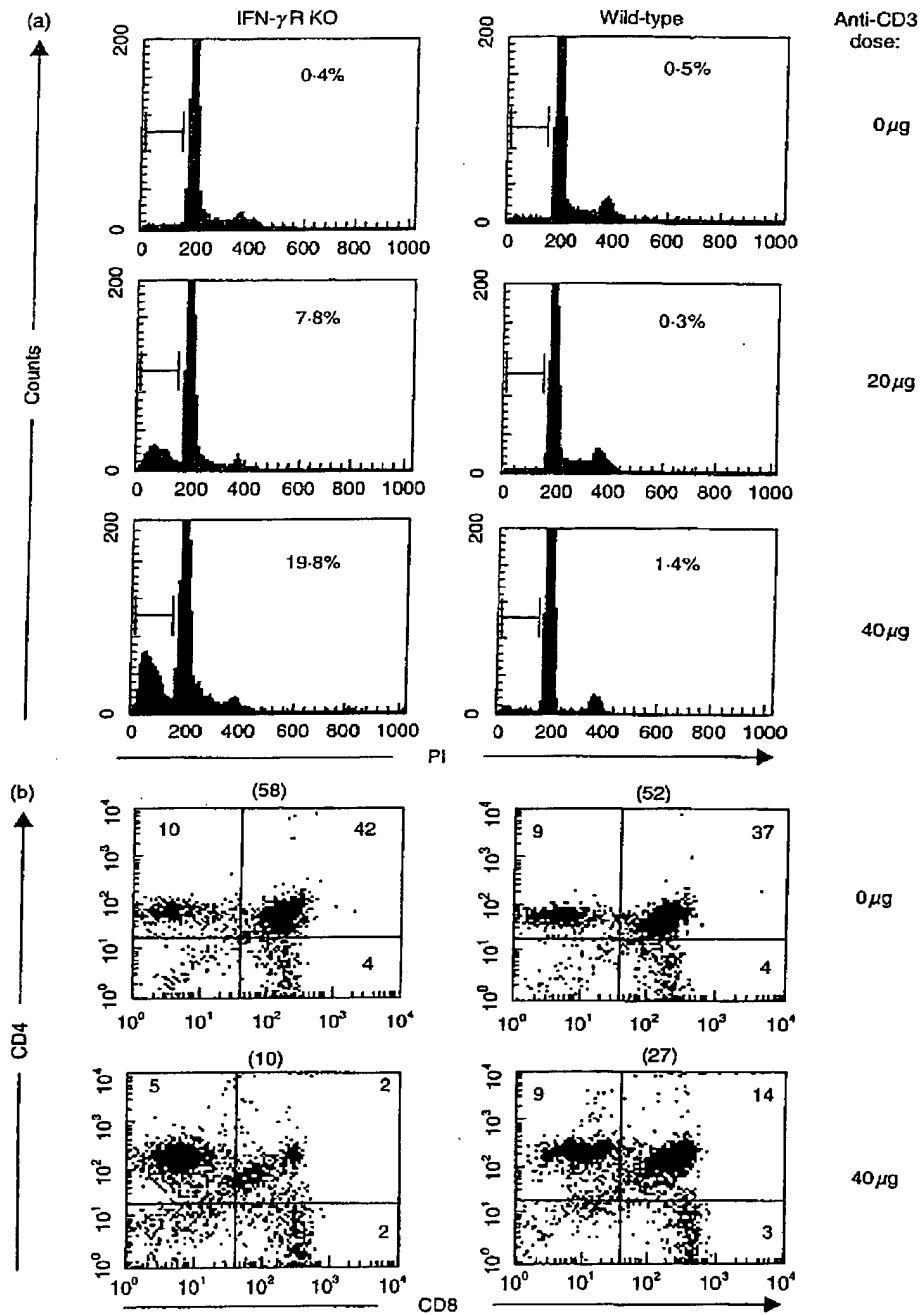
### Flow cytometric analyses and cell sorting

Thymuses were passed through cell strainers (Becton Dickinson Labware, Franklin Lakes, NJ, USA). Erythrocytes were removed by lysis with NH<sub>4</sub>Cl. Remaining cells were washed, resuspended in PBS and divided in aliquots of  $2 \times 10^5$  cells. For evaluation of apoptosis, thymocytes were fixed in 70% ice-cold ethanol, washed with Tris saline (pH 7.6) and stained with 0.5 mg/ml propidium iodide (PI, Sigma) containing 1 mg/ml RNase A (Boehringer). The hypoploid DNA was quantified by flow cytometric analysis using CELLQuest. For CD4/CD8 staining thymocytes were successively incubated for 30 min with a Fc receptor-blocking Ab (2-4G2, Pharmingen, San Diego, CA, USA; 1  $\mu$ g/ml) and 1  $\mu$ g FITC-conjugated rat anti-mouse CD8 and PE-conjugated rat anti-mouse CD4 (Caltag Laboratory, San Francisco, CA, USA). For flow cytometric analysis, the cells were resuspended in 0.5 ml of PBS containing 1% paraformaldehyde and 10 000 cells were analysed on a FACScan (Becton Dickinson, Mountain View, CA, USA). For flow cytometric cell sorting, the cells were resuspended in PBS + 5% FCS, and CD4<sup>+</sup>CD8<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> thymocytes were sorted to a purity of >99% using a FACS Vantage flow cytometer (Becton Dickinson) equipped with an argon laser.

## RESULTS

### Anti-CD3 Ab induces severe apoptosis of immature thymocytes in IFN- $\gamma$ R KO mice

We previously reported that IFN- $\gamma$ R KO mice are more sensitive than their wild-type counterparts to the anti-CD3-induced shock syndrome. They suffer from extensive tissue damage in liver and lungs and from severe apoptosis in the cortical region of the thymus. We here investigated the anti-CD3-induced apoptosis in the thymus in more detail. Preliminary experiments had established that following injection of anti-CD3 Ab, maximal thymic apoptosis occurs at 40–48 h. Accordingly, wild-type and IFN- $\gamma$ R KO mice were challenged with anti-CD3 Ab, and thymuses were removed 40 h later for flow-cytometric analysis of the number of cells with hypoploid DNA content (Fig. 1a). Both the magnitude of the apoptotic response and the sensitivity, in terms of minimal dose required to induce the response, were more elevated in the IFN- $\gamma$ R KO mice. Staining with anti-CD4 and anti-CD8



**Fig. 1.** Anti-CD3 Ab induces severe apoptosis in thymuses of IFN- $\gamma$ R KO mice. Groups of 3 IFN- $\gamma$ R KO and 3 wild-type mice were challenged with 0, 20 or 40  $\mu$ g anti-CD3 Ab. Forty hours post anti-CD3 challenge, thymocytes were analysed by flow cytometry for (a) their DNA content and (b) their staining pattern for CD4 and CD8. (a) PI fluorescence intensity: a representative histogram from one mouse out of three is shown. Value represents the percentage of apoptotic cells. (b) Thymocyte subpopulations in IFN- $\gamma$ R KO and wild-type mice. Each plot represents analysis of one mouse. The absolute number of each subpopulation as well as the total number of thymocytes (in parenthesis) are shown ( $\times 10^6$ ). Data are representative of three separate experiments.

antibodies revealed that apoptosis in the IFN- $\gamma$ R KO mice affected mainly CD4<sup>+</sup>CD8<sup>+</sup> T cells (Fig. 1b). The net number of these double-positive cells was reduced 20-fold, whereas single-positive cells showed only a 2-fold reduction.

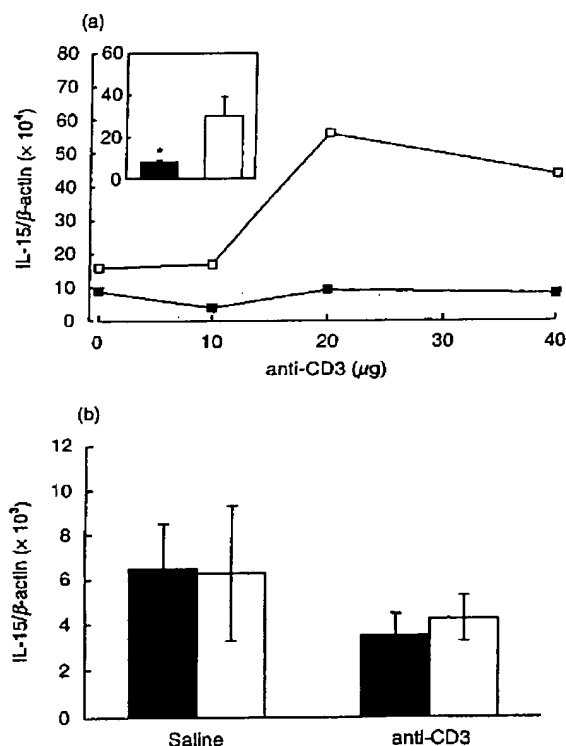
*Anti-CD3 Ab induces increased expression of IL-15 in the spleen of wild-type, but not of IFN- $\gamma$ R KO mice*

IFN- $\gamma$  is known to induce IL-15 expression in certain cells, including monocytes and macrophages [20]. Since our results indicated that IFN- $\gamma$  is a protective factor in anti-CD3 antibody-induced thymocyte apoptosis, we wanted to know whether induction of IL-15 by anti-CD3 antibody might depend on prior production of IFN- $\gamma$ . In sera of anti-CD3-treated IFN- $\gamma$ R KO and wild-type mice IL-15 protein was undetectable by ELISA (data not shown). Therefore, mRNA levels for IL-15 were determined by quantitative PCR in spleens and thymuses. As evident from Fig. 2a, spleens of IFN- $\gamma$ R KO or wild-type mice that were not injected with anti-CD3 antibody contained little mRNA of IL-15. Anti-CD3 challenge at a dose of 20  $\mu$ g was sufficient, in wild-type mice,

to induce a 4-fold increase in the expression level of IL-15 mRNA. As a contrast, in IFN- $\gamma$ R KO mice, IL-15 mRNA failed to be up-regulated following injection of as much as 40  $\mu$ g anti-CD3 Ab. Similar analysis on the thymuses of the mice revealed basal mRNA levels of IL-15 in saline-treated IFN- $\gamma$ R KO and wild-type mice (Fig. 2b). Anti-CD3 treatment failed to up-regulate IL-15 mRNA but rather down-regulated the expression to a similar level in IFN- $\gamma$ R KO and wild-type mice.

*Anti-IL-15 Ab enhances thymocyte apoptosis in anti-CD3 Ab-challenged wild-type mice and IL-15 protects IFN- $\gamma$ R KO mice from anti-CD3-induced thymocyte apoptosis*

The concordance of defective IL-15 expression in the spleen and increased thymic apoptosis in IFN- $\gamma$ R KO mice exposed to anti-CD3 Ab is suggestive for a causal relationship. In order to obtain more direct evidence for such a relationship, we first verified whether neutralization of endogenous IL-15 in wild-type mice could increase anti-CD3-induced apoptosis. To this end, wild-type mice were treated with neutralizing monoclonal rat anti-mouse IL-15 antibody or an irrelevant isotype control antibody, 5 h before challenge with anti-CD3 Ab. IFN- $\gamma$ R KO mice that were similarly challenged with anti-CD3 were included as positive controls. 40 h post challenge, thymuses were removed and apoptotic cells were enumerated by flow cytometry (Fig. 3a). In accordance with results of previous experiments, thymocyte apoptosis was more pronounced in IFN- $\gamma$ R KO mice than in wild-types not treated with anti-IL-15 Ab. Significantly, however, a level of apoptosis comparable in height to that in IFN- $\gamma$ R KO mice was seen in the wild-type mice that had received the anti-IL-15 Ab. CD4<sup>+</sup>, CD8<sup>+</sup> and CD4<sup>+</sup>CD8<sup>+</sup> subpopulations were analysed in these mice (Table 1). Differences between the net numbers in anti-IL-15 Ab- and irrelevant control-treated mice were again most evident for the double-positive subpopulation, and less pronounced for the CD4<sup>+</sup> and CD8<sup>+</sup> populations. The differential sensitivity in single- vs. double-positive thymocyte subpopulations could not be explained by differences in the expression of the IL-15R $\alpha$ . Thus, no significant differences in IL-15R $\alpha$  mRNA expression were observed between cell-sorted CD4<sup>+</sup>, CD8<sup>+</sup> and CD4<sup>+</sup>CD8<sup>+</sup> populations from either saline-treated IFN- $\gamma$ R KO or wild-type mice. Treatment with anti-CD3 Ab did not change these levels (data not shown).

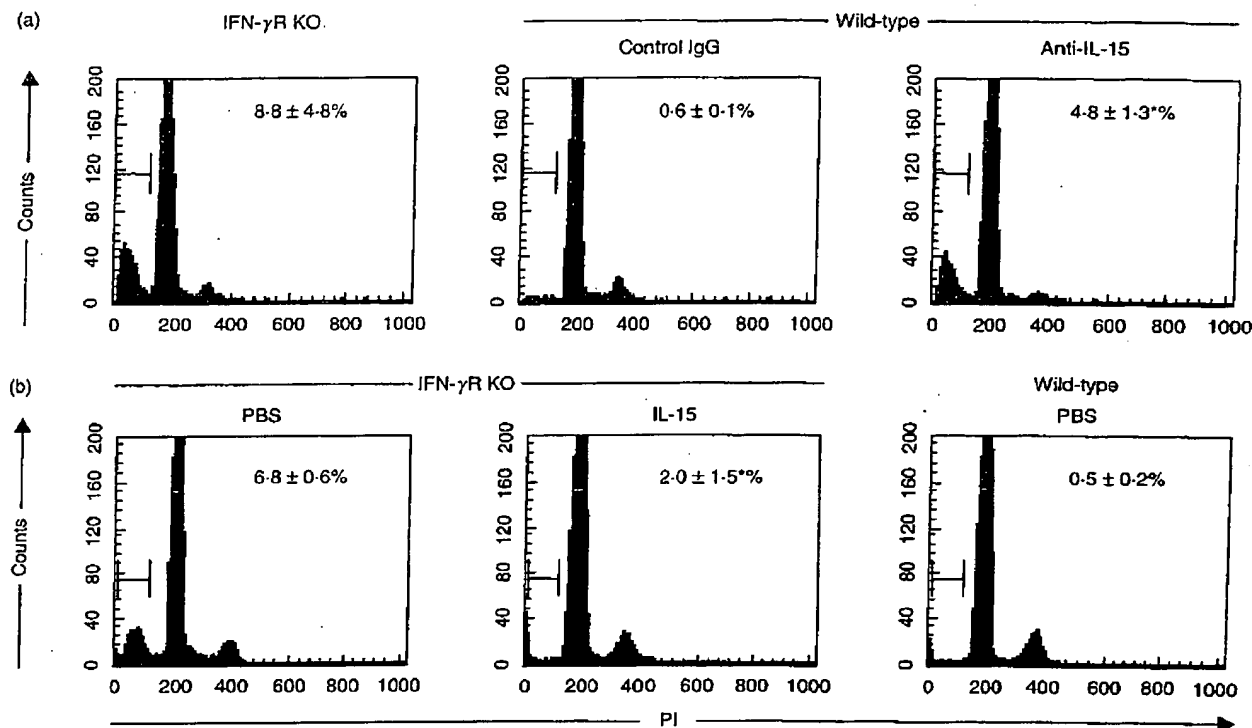


**Fig. 2.** Anti-CD3 induction of IL-15 in spleens of wild-type (□), but not of IFN- $\gamma$ R KO (■) mice. IFN- $\gamma$ R KO and wild-type mice were challenged with anti-CD3 Ab at the indicated dose. Six hours later, total RNA was extracted from spleens (a) or thymuses (b), and real-time PCR was performed for IL-15. (a) IL-15 mRNA expression in spleens of mice. Data of one mouse at each dose are shown. The inset shows a separate experiment performed with 20  $\mu$ g anti-CD3 Ab. Each bar represents the mean  $\pm$  S.E. of 3 mice. \* $P$  < 0.02 in comparison with wild-type mice (Mann-Whitney  $U$ -test). (b) IL-15 mRNA expression in thymuses of three IFN- $\gamma$ R KO and wild-type mice that had received 40  $\mu$ g anti-CD3 Ab or saline.

**Table 1.** Total numbers of thymic subpopulations after anti-CD3-challenge and treatment with anti-IL-15

Thymocytes	Mice and treatment†		
	IFN- $\gamma$ R KO	WT, clgG	WT, anti-IL-15
CD4 <sup>+</sup> CD8 <sup>+</sup>	2.35 $\pm$ 0.50	7.07 $\pm$ 1.10	3.61 $\pm$ 0.77*
CD4 <sup>+</sup> CD8 <sup>+</sup>	1.08 $\pm$ 0.44	11.96 $\pm$ 6.00	0.64 $\pm$ 0.17*
CD4 <sup>+</sup> CD8 <sup>+</sup>	0.84 $\pm$ 0.16	2.37 $\pm$ 0.26	1.40 $\pm$ 0.33
CD4 <sup>+</sup> CD8 <sup>+</sup>	0.32 $\pm$ 0.07	1.39 $\pm$ 0.40	0.42 $\pm$ 0.09

†The number of cells (mean  $\pm$  S.E.,  $\times 10^{-6}$  cells/organ) from 4 mice in each group are shown. Mice were treated as described in the legend of Fig. 3. WT, wild-type mice. \* $P$  < 0.05 in comparison with control IgG (clgG)-treated wild-type mice (Mann-Whitney  $U$ -test).



**Fig. 3.** Anti-IL-15 Ab increases anti-CD3-induced apoptosis in (a) wild-type mice and (b) IL-15 protects IFN- $\gamma$  KO mice from anti-CD3-induced apoptosis. (a) Two groups of wild-type mice ( $n = 4$ ) were treated with anti-IL-15 Ab or control IgG. Five hours later, these mice and an additional group of 4 IFN- $\gamma$  KO mice, were challenged with 20  $\mu$ g anti-CD3 Ab. Forty hours post anti-CD3-challenge, apoptosis in the thymuses was measured with the use of the DNA dye PI. Each histogram represents analysis of a single thymus. Values indicate the average percentages of apoptotic cells (mean  $\pm$  S.E.) for 4 mice in each group. \* $P < 0.05$  in comparison with control IgG-treated wild-type mice (Mann-Whitney  $U$ -test). (b) Two groups of IFN- $\gamma$  KO mice ( $n = 3$ ) were injected with 20  $\mu$ g anti-CD3-Ab and two injections of either 15  $\mu$ g IL-15 in 100  $\mu$ l PBS, or PBS as control, at 6 h and 18 h post anti-CD3 challenge. Wild-type mice that similarly received anti-CD3 and PBS were also included. Thymocyte apoptosis was analysed with PI. Each histogram represents analysis of a single thymus. Values indicate the average percentages of apoptotic cells (mean  $\pm$  S.E.) for 3 mice in each group. \* $P < 0.05$  in comparison with PBS-treated IFN- $\gamma$  KO mice (Mann-Whitney  $U$ -test).

In a following experiment, we verified whether the increased susceptibility of IFN- $\gamma$  KO mice to anti-CD3-induced apoptosis could be rescued by systemic IL-15 administration. IFN- $\gamma$  KO mice were treated with two injections of 15  $\mu$ g IL-15 at 6 h and 18 h post anti-CD3 challenge, and effects on thymocyte apoptosis were analysed at 40 h. As can be seen from the PI-staining pattern in Fig. 3b, treatment with IL-15 inhibited anti-CD3-induced thymocyte apoptosis in IFN- $\gamma$  KO mice. The inhibition of thymocyte apoptosis by IL-15 was also evident on the total numbers of immature CD4<sup>+</sup>CD8<sup>+</sup> T cells (the mean  $\pm$  S.E.,  $\times 10^6$ /thymus for anti-CD3-challenged IFN- $\gamma$  KO mice treated with PBS ( $n = 3$ ) and IL-15 ( $n = 3$ ) were, respectively, 1.3  $\pm$  0.5 and 3.1  $\pm$  0.2;  $P < 0.05$ , Mann-Whitney  $U$ -test).

### DISCUSSION

An important aspect of the anti-CD3 syndrome is the occurrence of extensive apoptosis of mainly immature T lymphocytes in the thymus [5]. A key cytokine in the syndrome appeared to be IFN- $\gamma$  as we previously reported that IFN- $\gamma$  KO mice are much more

sensitive than their wild-type counterparts [4]. The results indicate that IFN- $\gamma$  produced during the reaction exerts a protective effect. Here, we report that another key cytokine in the syndrome is IL-15. Injection of anti-CD3 Ab was found to induce increased expression of IL-15 mRNA in spleen but not in thymus of wild-type mice. Induction of IL-15 mRNA was absent in IFN- $\gamma$  KO mice, demonstrating that the TCR-triggered production of IL-15 depends on IFN- $\gamma$  signalling. Administration of anti-CD3 Ab in mice is well known to induce the release of several Th1 and Th2 related cytokines, including IFN- $\gamma$ , in the circulation [reviewed in 4]. IFN- $\gamma$  is probably the most potent monocyte/macrophage activating factor [21]. Monocytes/macrophages are an important source of IL-15 and its expression is increased by various macrophage-activating stimuli including IFN- $\gamma$  [22]. It is therefore tempting to speculate that in the anti-CD3 system, IL-15 may be produced by macrophages from the spleen as a result of TCR-triggered IFN- $\gamma$ .

IL-15 is well known to exert an anti-apoptotic effect on mature T cells [13–15]. In anti-CD3 Ab-challenged mice however, apoptosis was found to affect mainly immature (CD4<sup>+</sup>CD8<sup>+</sup>)

thymocytes and only to a lesser extent mature T cells. Treatment of wild-type mice with anti-IL-15 Ab perfectly mimicked the results obtained in IFN- $\gamma$ R KO mice and similarly resulted in an increased apoptosis of mainly immature thymocytes. In the reversed approach, administration of IL-15 in IFN- $\gamma$ R KO mice rescued immature thymocytes from anti-CD3-induced apoptosis. Our results therefore indicate an anti-apoptotic effect of IL-15 that encompasses not only mature but also immature T cells. Immature thymocytes were found to express levels of IL-15R $\alpha$ , comparable with those of mature thymocytes, indicating that their sensitivity to anti-CD3-triggered apoptosis reflects the well known sensitivity of CD4<sup>+</sup>CD8<sup>+</sup> immature T cells for TCR-triggered cell death rather than deficiency in IL-15 receptor expression. The anti-apoptotic activity of IL-15 on immature T cells and its defective induction by anti-CD3 Ab in the absence of IFN- $\gamma$  signalling, provide an explanation for the exaggerated T cell apoptosis in thymuses of IFN- $\gamma$ R KO mice following TCR/CD3 aggregation. Furthermore, the anti-apoptotic activity of IL-15 on immature T cells may explain the reduced thymus cell content previously observed in IL-15 [11] and in IL-15 receptor knock-out mice [23].

Apoptosis of immature T cells has to be seen as an important event in central tolerance, i.e. a process in which autoreactive T cells are eliminated in the thymus. Our data raise the possibility that IL-15 plays a role in this process by setting the sensitivity level to the apoptotic signals. Hence, systemic IL-15 may be involved not only in regulation of peripheral tolerance, as has been suggested by others and ourselves [12,14], but also in regulation of central tolerance. Interestingly, IL-15 has been shown to act *in vitro* and *in vivo* as a costimulator of IFN- $\gamma$  production by cells of the innate immune system [24,25]. The present finding that TCR-triggered IFN- $\gamma$  induces or potentiates IL-15 production, implies the possible existence of a self-amplifying feedback mechanism which tends to perpetuate production of both cytokines. If not kept under control, this vicious circle could unduly protect autoreactive T cells in the periphery but also in the thymus against apoptosis, and thereby maintain or trigger autoimmunity. Hence, the increased levels of IL-15 that accompany autoimmune and chronic inflammatory disorders such as rheumatoid arthritis and Crohn's disease [26–28] may need to be seen not merely as symptomatic of the inflammatory condition but rather as a link in the pathogenesis by compromising peripheral and central tolerance to self.

#### ACKNOWLEDGEMENTS

The authors are indebted to Dr S. Huang for providing the IFN- $\gamma$ R KO mice. This work was supported by grants from the Fund for Scientific Research Flanders (FWO – Vlaanderen), from the Regional Government of Flanders (GOA Program), and from the Belgian Federal Government (Inter-University Network for Fundamental Research – IUAP). P.M. is a postdoctoral research fellow of the FWO.

#### REFERENCES

- Hirsch R, Gress RE, Pluznik DH, Eckhaus M, Bluestone JA. Effects of *in vivo* administration of CD3 monoclonal antibody on T cell function in mice. II. *In vivo* activation of T cells. *J Immunol* 1989; **142**:737–43.
- Alegre M, Vandenabeele P, Depierreux M *et al.* Cytokine release syndrome induced by the 145–2C11 anti-CD3 monoclonal antibody in mice: prevention by high doses of methylprednisolone. *J Immunol* 1991; **146**:1184–91.
- Ferran C, Dy M, Sheehan K *et al.* Cascade modulation by anti-tumor necrosis factor monoclonal antibody of interferon- $\gamma$ , interleukin-3 and interleukin-6 release after triggering of the CD3/T cell receptor activation pathway. *Eur J Immunol* 1991; **21**:2349–53.
- Matthys P, Froyen G, Huang S *et al.* Interferon- $\gamma$  receptor-deficient mice are hypersensitive to the anti-CD3-induced cytokine release syndrome and thymocyte apoptosis: Protective role of endogenous nitric oxide. *J Immunol* 1995; **155**:3823–9.
- Shi Y, Bissonnette RP, Parfrey N, Szalay M, Kubo RT, Green DR. *In vivo* administration of monoclonal antibodies to the CD3 T cell receptor complex induces cell death (apoptosis) in immature thymocytes. *J Immunol* 1991; **146**:3340–6.
- Grabstein KH, Eisenman J, Shanebeck K *et al.* Cloning of a T-cell growth factor that interacts with the  $\beta$ -chain of the interleukin-2 receptor. *Science* 1994; **264**:965–8.
- Bamford RN, Battista AP, Burton JD, Sharma H, Waldmann TA. Interleukin (IL) 15/IL-T production by the adult T-cell leukemia cell line HuT-102 is associated with a human T-cell lymphotropic virus type 1 region/IL-15 fusion message that lacks many upstream AUGs that normally attenuates IL-15 mRNA translation. *Proc Natl Acad Sci USA* 1994; **93**:2897–909.
- Bamford RN, Grant AJ, Burton JD *et al.* The interleukin (IL) 2 receptor  $\beta$  chain is shared by IL-2 and a cytokine, provisionally designated IL-T, that stimulates T-cell proliferation and the induction of lymphokine-activated killer cells. *Proc Natl Acad Sci USA* 1994; **91**:4940–4.
- Armitage RJ, Macduff BM, Eisenman J, Paxton R, Grabstein KH. IL-15 has stimulatory activity for the induction of B cell proliferation and differentiation. *J Immunol* 1995; **154**:483–90.
- Carson WE, Giri JG, Lindenmann MJ *et al.* Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor. *J Exp Med* 1994; **180**:1395–403.
- Kennedy MK, Glaccum M, Brown SN *et al.* Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. *J Exp Med* 2000; **191**:771–80.
- Dooms H, Van Belle T, Desmedt M, Rottiers P, Grooten J. Interleukin-15 redirects the outcome of a tolerizing T cell stimulus from apoptosis to anergy. *Blood* 2000; **96**:1006–12.
- Dooms H, Desmedt M, Vancaeneneghem S, Rottiers P, Goossens V, Fiers W. Quiescence-inducing and antiapoptotic activities of IL-15 enhance secondary CD4<sup>+</sup> T cell responses to antigen. *J Immunol* 1998; **161**:2141–50.
- Marks-Konczalik J, Dubois S, Losi JM *et al.* IL-2-induced activation-induced cell death is inhibited in IL-15 transgenic mice. *Proc Natl Acad Sci USA* 2000; **97**:11445–50.
- Bulfone-Paus S, Ungureanu D, Pohl T *et al.* Interleukin-15 protects from lethal apoptosis *in vivo*. *Nat Med* 1997; **3**:1124–8.
- Bulfone-Paus S, Bulanova E, Pohl T *et al.* Death deflected: IL-15 inhibits TNF- $\alpha$ -mediated apoptosis in fibroblasts. *FASEB J* 1999; **13**:1575–85.
- Huang S, Hendriks W, Althage A *et al.* Immune response in mice that lack the interferon- $\gamma$  receptor. *Science* 1993; **259**:1742–5.
- Matthys P, Mitera T, Heremans H, Van Damme J, Billiau A. Anti-IFN- $\gamma$  and anti-IL-6 antibodies affect staphylococcal enterotoxin B-induced weight loss, hypoglycemia and cytokine release in D-galactosamine-sensitized and unsensitized mice. *Infect Immun* 1995; **63**:1158–64.
- Overbergh L, Valckx D, Waer M, Mathieu C. Quantification of murine cytokine mRNAs using real time quantitative reverse transcriptase PCR. *Cytokine* 1999; **11**:305–12.
- Waldmann TA, Tagaya Y. The multifaceted regulation of interleukin-15 expression and the role of this cytokine in NK cell differentiation and host response to intracellular pathogens. *Ann Rev Immunol* 1999; **17**:19–49.
- Billiau A. Interferon- $\gamma$ : biology and role in pathogenesis. *Adv Immunol* 1996; **62**:61–130.

- 22 Doherty TM, Seder RA, Sher A. Induction and regulation of IL-15 expression in murine macrophages. *J Immunol* 1996; **156**:735-41.
- 23 Lodolce JP, Boone DL, Chai S *et al*. IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. *Immunity* 1998; **9**:669-76.
- 24 Fehniger TA, Yu H, Cooper MA, Suzuki K, Shah MH, Caligiuri MA, Cutting Edge. IL-15 costimulates the generalized Shwartzman reaction and innate immune IFN- $\gamma$  production in vivo. *J Immunol* 2000; **164**:1643-7.
- 25 Carson WE, Ross ME, Baiocchi RA *et al*. Endogenous production of interleukin 15 by activated human monocytes is critical for optimal production of interferon- $\gamma$  by natural killer cells in vitro. *J Clin Invest* 1995; **96**:2578-82.
- 26 McInnes IB, Leung BP, Sturrock RD, Field M, Liew FY. Interleukin-15 mediates T cell-dependent regulation of tumor necrosis factor- $\alpha$  production in rheumatoid arthritis. *Nat Med* 1997; **3**:189-95.
- 27 McInnes IB, Liew FY. Interleukin 15: a proinflammatory role in rheumatoid arthritis synovitis. *Immunol Today* 2001; **19**:75-9.
- 28 Kirman I, Nielsen OH. Increased numbers of interleukin-15-expressing cells in ulcerative colitis. *Am J Gastroenterol* 1996; **91**:1789-94.